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Effects of anabolic agents on protein breakdown in L6 myoblasts

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1. Protein degradation in rat L6 myoblasts is inhibited by high concentrations of insulin as well as by foetal bovine serum and bovine colostrum, mixtures rich in growth-factor activity. 2. Growth factors achieve maximal effects within 2h after addition to the cell cultures, but these diminish with time. Indeed, during incubations greater than 12h, foetal calf serum actually stimulates protein breakdown. The changed response, however, is not due to the depletion of growth factors from serum. 3. Protein breakdown is stimulated by dexamethasone by a process that takes several hours to be expressed, but is more pronounced over a 4h measurement period than over 18h. The glucocorticoid response is prevented by insulin or by cycloheximide. 4. Anabolic agents such as trenbolone, diethylstilboestrol and testosterone do not alter rates of intracellular protein breakdown and do not interfere with the glucocorticoid-induced catabolic response. 5. The results are consistent with anabolic steroids and related agents acting indirectly on muscle, perhaps via altering concentrations of growth factors of the somatomedin type.

Net accumulation of protein is essential for growth as well as being an important goal during treatment of catabolic states after injury, trauma or muscle-wasting diseases. Anabolic steroids and related agents have been shown to increase growth rates, especially for female or castrated male animals, and also to improve the efficiency by which nutrients are converted into muscle protein (van Weerden & Grandadam, 1976; Heitzman, 1976, 1979; VanderWal, 1976; Vernon & Buttery, 1978). The mode of action of these agents has not been fully clarified, although it has been suggested that trenbolone acetate (19-norandrosta-4,9,11-trien-3-one 17-acetate) and other anabolic steroids may modify the responses of endogenous hormones and thus change growth rates. Increases in plasma insulin and somatomedins, as well as decreases in thyroxine concentrations, occur after implantation of trenbolone acetate or the substituted resorcyclic acid lactone, zeranol (Donaldson *et al.*, 1981; Wangsness *et al.*, 1981). These effects have led to the proposal that one or more of these secondary hormone changes may be important. Rates of both muscle protein synthesis and breakdown are decreased after injection of trenbolone acetate into female rats (Vernon & Buttery, 1978), but the accumulation of carcass nitrogen is greater than in control animals because the rate of protein breakdown is depressed more than the rate of protein synthesis.

Effects of anabolic steroids on protein turnover have apparently not been examined on isolated muscle or cultured myoblasts, although treatment of animals with thyroxine (DeMartino & Goldberg, 1978; Flaim *et al.*, 1978) or glucocorticoids (Goldberg, 1969) produces catabolic effects when muscle is subsequently incubated *in vitro*. These effects, together with the well-established ability of insulin to stimulate protein synthesis and inhibit protein breakdown in isolated muscle and cultured cells (Goldberg & St. John, 1976; Ballard, 1980; Ballard *et al.*, 1980*a*), are consistent with the hypothesis that anabolic steroids act via other hormones. We have demonstrated the inhibition of protein breakdown by insulin-like growth factors and epidermal growth factor in several cell lines (Ballard *et al.*, 1980*a*,*b*). In the present paper we have examined the effects of growth factors and anabolic agents on protein breakdown in a myoblast cell line in order to characterize the responses and to distinguish direct effects from those that may occur indirectly *in vivo*.

Experimental

Materials

Rat L6 myoblasts were a non-fusing line kindly provided by Dr. J. M. Gunn, Texas A & M University, College Station, TX, U.S.A. Foetal-calf serum (batch 29101829) was purchased from Flow Laboratories, Stanmore, N.S.W., Australia; amino

acids and vitamins for the preparation of media, together with thyroxine, tri-iodothyronine and diethylstilboestrol, were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; Eagle's Minimal Essential Medium was obtained from Gibco, Grand Island, NY, U.S.A. The insulin used was Actrapid from Novo Industri A/S, Copenhagen, Denmark, and epidermal growth factor was a gift from Dr. J. Koch, CSIRO Molecular and Cellular Biology Unit, North Ryde, N.S.W., Australia. Details of the bovine colostrum (batch 2) have been given previously (Ballard *et al.*, 1982). L-[4,5-^{3}H]Leucine (40–60 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. L-[3,5-^{3}H]Tyrosine (40–60 Ci/mmol) and L-[2,5-^{3}H]histidine (40–60 Ci/mmol) were purchased from Amersham Corp., Sydney, N.S.W., Australia. Dexamethasone sodium phosphate was supplied by Merck, Sharp and Dohme (Aust.) Pty. Ltd., Granville, N.S.W., Australia, and testosterone was from Fluka A.G., Buchs, Switzerland. Trenbolone acetate was a gift from Hoechst Roussel Pharmaceuticals Pty. Ltd., Castle Hill, N.S.W., Australia. Penicillin G and streptomycin sulphate were from Glaxo Pty. Ltd., Boronia, Victoria, Australia; gentamycin was from Schering Corp., Kenilworth, NJ, U.S.A., and fungizone was from E. R. Squibb and Sons, Princeton, NJ, U.S.A.

Measurements of protein breakdown

The basic method for measuring rates of protein breakdown has been described previously (Ballard *et al.*, 1980*a*). Briefly, each confluent monolayer in a 24-well Costar dish was labelled for 16 h in 1 ml of growth medium (Eagle's Minimal Essential Medium containing 50 μl of foetal bovine serum, 50 μg of gentamycin, 100 μg of streptomycin, 60 μg of penicillin, 1 μg of fungizone), but with 1 μCi of [^{3}H]leucine added and non-radioactive leucine omitted. This medium was modified for tyrosine labelling by the addition of 0.4 μmol of leucine and 1 μCi of [^{3}H]tyrosine and the omission of radioactive leucine and non-radioactive tyrosine. For histidine labelling, non-radioactive histidine was omitted from the growth medium and 1 μCi of [^{3}H]histidine was added. A 3-day labelling period was used for time-course experiments and those where degradation was measured over 18 h. After completion of the labelling period, the medium was removed, and each monolayer was washed twice with growth medium with serum omitted and left for a 3 h chase period in a similar medium. The degradation period commenced by the replacement of chase medium with 0.9 ml of degradation medium, which was similar to the chase medium but contained an additional 5 μmol of leucine, 0.5 μmol of tyrosine or 5 μmol of histidine as chase for the respective labelling amino acid. Effectors were diluted as required in chase medium and 100 μl was added to each monolayer at this time. Steroids were dissolved in dimethyl sulphoxide and diluted before addition so that the final concentration of this solvent was 0.1%; a similar concentration was added to the respective controls. At the completion of the degradation period, radioactivity was measured in amino acids and protein in the medium and in cell protein as described by Ballard *et al.* (1980*a*). Rates of protein breakdown were expressed as the percentage of the total radioactivity in the amino acid fraction.

Release of protein into the medium is also monitored in order to provide an index of cell viability. For L6 myoblasts the radioactivity in medium proteins is typically 2–4% of total radioactivity over a 4 h degradation period or 10% over an 18 h degradation period. Although the former percentage is not altered by any agents tested here, the inclusion of serum or insulin in the degradation medium slightly decreases, and cycloheximide in the absence of serum approximately doubles, protein loss when incubations are extended to 18 h.

Results

Choice of labelling amino acid and chase conditions

Although leucine is frequently used to label protein in measurements of protein turnover in cultured cells, it would not be suitable for myoblast experiments if it was metabolized as extensively as reported for muscle *in vivo* (Goldberg & Chang, 1978). Analyses of radioactivity in cells and medium at the completion of an 18 h degradation period, however, showed that approx. 98% and 95% respectively was present in leucine. The remaining 5% of the radioactivity in the amino acid fraction in the medium was eluted with the void volume during chromatography using the protocol for acidic plus neutral amino acids for the Jeol 6AH amino acid analyser (results not shown). This radioactivity was presumably ^3H_2O, because it was lost on freeze-drying. A comparable proportion of the radioactivity in the medium in a [^{3}H]tyrosine-labelling experiment was also eluted in the column void volume.

The rates of protein breakdown in Eagle's Minimal Essential Medium were similar whether cell protein was prelabelled with leucine, tyrosine or histidine (Table 1). Inclusion of 5% foetal bovine serum, 5 mM-NH_4Cl, 1 μM-insulin or 0.1 mM-cycloheximide produced equivalent inhibitory effects on protein breakdown for leucine- and tyrosine-labelled cells. The effects were generally less when histidine had been used as labelling precursor (Table 1). The extent of protein labelling was very different, however, with tyrosine producing about 15% and histidine 3% of that obtained with [^{3}H]leucine. This decrease in labelling would decrease the precision of

Table 1. *Rates of protein breakdown in L6 myoblasts measured after prelabelling with different ^3H-labelled amino acids*

Protein breakdown was measured over a 4h period after prelabelling cell protein with the indicated amino acids. Values are means ± S.E.M. for six determinations, with the percentage inhibition of the control rate in Minimal Essential Medium shown in parentheses.

Additions	Protein degraded (%) after labelling with: Leucine	Histidine	Tyrosine
None	8.70 ± 0.04	8.24 ± 0.06	8.38 ± 0.08
Serum (5%)	7.29 ± 0.12 (16%)	7.54 ± 0.27 (8%)	6.80 ± 0.15 (19%)
NH_4Cl (5 mM)	5.95 ± 0.13 (32%)	6.66 ± 0.23 (19%)	5.86 ± 0.12 (30%)
Insulin (1 μM)	6.52 ± 0.19 (25%)	6.23 ± 0.25 (24%)	5.81 ± 0.10 (31%)
Cycloheximide (0.1 mM)	6.30 ± 0.12 (28%)	6.40 ± 0.17 (22%)	5.94 ± 0.11 (29%)

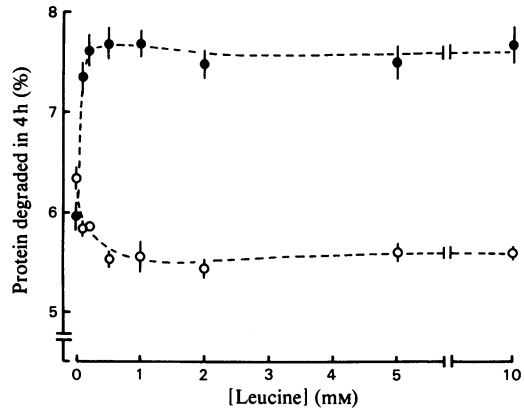

Fig. 1. *Effect of leucine concentration on the percentage of leucine-labelled protein degraded over a 4h period*

The control medium was Eagle's Minimal Essential Medium without (●) or with (○) 0.1 mM-cycloheximide. For further details see the text. Values are means ± S.E.M. for six determinations at each leucine concentration.

degradation measurements unless removal of unincorporated radioactivity was more carefully monitored and much longer counting times were used, especially for the determination of radioactivity in the amino acid fraction in the medium.

For reliable measurements of protein breakdown it is essential that the labelled leucine produced from catabolism of intracellular proteins is not reincorporated into protein. Whereas this aim can be achieved by inhibiting protein synthesis, effective drugs such as cycloheximide usually themselves inhibit protein breakdown and may also mask effects of other agents. This situation occurs in L6 myoblasts (Table 1, Fig. 1). An alternative means of decreasing reincorporation is to increase the concentration of the amino acid in the chase and degradation media so that labelled amino acid released during protein breakdown mixes with a large pool. The effectiveness of this approach is illustrated in Fig. 1, which shows that leucine concentrations above 0.2 mM give rise to a constant apparent rate of protein breakdown. This plateau rate is considerably higher than found in the absence of added leucine (Fig. 1), implying that the reutilization occurring under such conditions can be prevented by the inclusion of leucine in the medium. We have chosen a concentration of 5 mM-leucine for use in all subsequent experiments. It should be noted that the inclusion of cycloheximide in the medium together with different concentrations of leucine also gives a constant rate of protein breakdown except at very low leucine concentrations. Indeed, in the absence of added leucine the measured rate of protein breakdown in the presence of cycloheximide is actually higher than that observed without cycloheximide. This finding further suggests that the reutilization of label occurring at low leucine concentrations is effectively prevented by the protein-synthesis inhibitor. Although we have no evidence why the apparent rate of protein breakdown in the presence of cycloheximide decreases as the leucine concentration is increased from zero to 0.2 mM (Fig. 1), it is possible that the inhibitory effect of cycloheximide on protein breakdown is independent of any effect on protein synthesis and requires a complete amino acid mixture to be expressed fully.

Effect of growth factors on protein breakdown

The inclusion of 5% serum in the medium inhibits protein breakdown in myoblasts by about 18% provided that the measurement is made over a 2h incubation period (Fig. 2). The percentage inhibition decreases as the measurement period is increased, so that beyond 12h the protein-breakdown rate is actually stimulated by serum. Results obtained with insulin are similar, although a lesser inhibitory effect still persists over a 24h measurement period. We have also performed comparable experiments with epidermal growth factor. This protein, at concentrations between 10 pM and 10 nM, does not affect rates of protein breakdown in L6 myoblasts in measurements over either 4h or 18h periods. The progressive loss of the inhibitory effect of serum or insulin does not occur with all agents that inhibit protein breakdown. Thus 5 mM-NH_4Cl added to-

gether with 5% foetal bovine serum produces a relatively constant 30–35% inhibition from 2 to 24h (Fig. 2). Moreover, cycloheximide addition leads to a gradually increasing degree of inhibition from less than 10% after 2h to about 30% at 4h or longer periods (results not shown).

It is possible that the decreased inhibitory effect with time shown for serum or insulin (Fig. 2) is caused by the metabolism of the active agents by the cell monolayer. To test this proposal we produced conditioned media by incubating unlabelled monolayers for 18h with the normal degradation medium or medium containing 5% foetal bovine serum. The relative effect of serum on protein breakdown in myoblasts was the same whether fresh or conditioned serum was used (Table 2). We note, however, that protein breakdown in cells incubated with conditioned medium was about 15% lower than that observed with fresh medium.

Although insulin gives rise to a similar inhibition of protein breakdown to that found with 5% serum, the hormone is only effective at high concentrations. A slight effect can be demonstrated at 10nM-insulin, but the maximum response requires at least 100nM (Fig. 3).

Protein breakdown in L6 myoblasts is inhibited at low concentrations of serum or bovine colostrum (Fig. 3). Both these mixtures provide sufficient factors to permit the growth of L6 myoblasts in

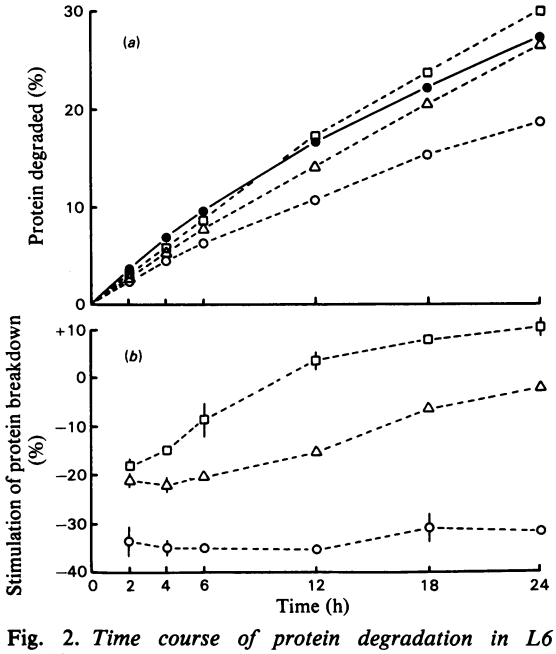

Fig. 2. *Time course of protein degradation in L6 myoblasts*

Measurements were done, as described in the test, in Eagle's Minimal Essential Medium containing 5mM-leucine (●), or in similar medium with additional 1μM-insulin (△), 5% foetal bovine serum (□) or 5% foetal bovine serum plus 5mM-NH_4Cl (○). (*a*) Degradation rates; (*b*) mean percentage stimulation of protein breakdown at each time period, ±S.E.M. for four determinations.

Table 2. *Factors in foetal bovine serum that inhibit protein breakdown and are retained after 18h preincubation*

Eagle's Minimal Essential Medium with or without 5% foetal bovine serum and containing 5mM-leucine was conditioned by exposure to L6 myoblasts for 18h. These media were removed and added to other cells previously labelled with [^{3}H]leucine for 18h. Degradation of prelabelled protein was followed for 4h as described in the Experimental section. Other prelabelled cells were incubated with fresh medium with or without fresh 5% foetal bovine serum. Values are means ± S.E.M. for four wells.

Medium	Protein degraded (%)	Effect of serum (%)
Fresh medium	8.32 ± 0.15	—
Fresh medium plus 5% fresh serum	6.60 ± 0.10	32
Conditioned medium	7.15 ± 0.10	—
Conditioned medium containing 5% conditioned serum	5.83 ± 0.03	18

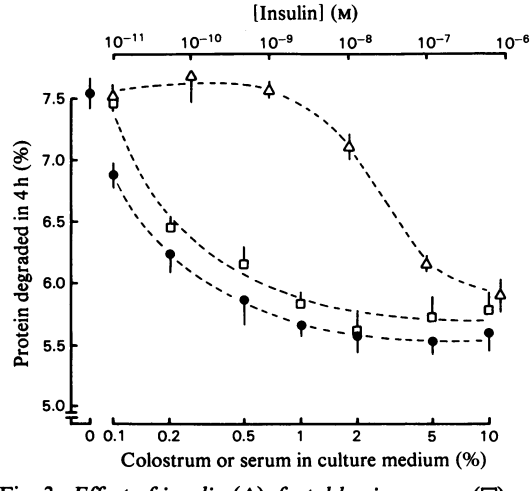

Fig. 3. *Effect of insulin* (△), *foetal bovine serum* (□) *and bovine colostrum* (●) *on protein breakdown measured over a 4h period*

Values are means ± S.E.M. for three determinations at each concentration. For further details see the text.

Table 3. *Effect of dexamethasone on protein breakdown*
Protein degradation was measured over a 4h period in myoblasts labelled with [^{3}H]leucine and pre-exposed for 3h or 24h as indicated below. The control medium was Eagle's Minimal Essential Medium containing 5mM-leucine. Values are means ± S.E.M. for the numbers of determinations shown in parentheses.

Dexamethasone	Preincubation	Protein degraded (%)
0	—	8.71 ± 0.06 (6)
100nM	—	8.97 ± 0.10 (6)
1μM	—	9.17 ± 0.15 (6)
1nM	3h	8.71 ± 0.03 (3)
10nM	3h	9.07 ± 0.07 (3)
100nM	3h	9.92 ± 0.25 (3)
1μM	3h	10.80 ± 0.13 (3)
100nM	24h	9.84 ± 0.14 (3)

Table 4. *Inhibition of dexamethasone effects by cycloheximide, NH_4Cl or insulin*
Protein breakdown was measured in L6 myoblasts incubated in Minimal Essential Medium containing 5mM-leucine together with the additions listed. All effectors were present for 3h before as well as during the measurement period. Values are means ± S.E.M. for the numbers of dishes indicated in parentheses.

	Protein degraded (%)	
Additions	4h	18h
None	8.68 ± 0.21 (6)	21.05 ± 0.22 (4)
Insulin (1μM)	6.04 ± 0.07 (3)	19.14 ± 0.44 (3)
Cycloheximide (0.1μM)	4.71 ± 0.03 (3)	13.50 ± 0.07 (4)
NH_4Cl (10mM)	5.47 ± 0.08 (3)	15.97 ± 0.28 (4)
Dexamethasone (1μM)	10.76 ± 0.10 (6)	22.65 ± 0.22 (4)
Dexamethasone (1μM) + insulin (1μM)	6.22 ± 0.12 (3)	18.43 ± 0.04 (3)
Dexamethasone (1μM) + cycloheximide (0.1μM)	4.74 ± 0.03 (3)	13.36 ± 0.10 (3)
Dexamethasone (1μM) + NH_4Cl (10mM)	6.16 ± 0.02 (3)	16.87 ± 0.15 (4)

media without the addition of other proteins (results not shown), and both inhibit protein breakdown when added at 0.2%.

Effects of steroids on protein breakdown

The addition of dexamethasone to L6 myoblasts at the beginning of the degradation period produced a slight stimulus of protein breakdown (Table 3). However, inclusion of the synthetic glucocorticoid during the 3h chase as well as throughout the 4h measurement period resulted in up to 24% stimulation. Under these conditions a half-maximal response occurred between 10 and 100nM-dexamethasone. Extension of the preincubation period from 3 to 24h did not increase the effect (Table 3). When the measurement period was 18h, dexamethasone gave rise to a much smaller stimulus of protein breakdown (Table 4). Insulin and cycloheximide completely blocked the dexamethasone response when it was measured either over a 4h or an 18h period, whereas 10mM-NH_4Cl partially decreased the dexamethasone effect (Table 4). In a parallel series of experiments, different concentrations of diethylstilboestrol, testosterone and trenbolone added during the 3h chase as well as during the 4h measurement period had no effect on protein breakdown in L6 myoblasts (Table 5). Similarly the anabolic agents did not modify the dexamethasone-induced activity. Moreover, trenbolone had no effect on protein breakdown measured over 18h. Although not shown in Table 5, the control rate of protein breakdown over 4h was not altered by the addition of 1μM-thyroxine or 0.1μM-tri-iodothyronine even when the thyroid hormones were also present during the 3h chase or during labelling and chase periods.

Table 5. *Effect of anabolic agents on protein breakdown*
Protein breakdown was measured as described in the legend of Table 4, except that all dishes contained 0.1% dimethyl sulphoxide.

		Protein degraded (%)	
Degradation period	Anabolic agent	No additions	+ 1μM-dexamethasone
4h	None	8.79 ± 0.08 (6)	10.72 ± 0.10 (6)
	Diethylstilboestrol		
	(1nM)	8.83 ± 0.08 (3)	11.18 ± 0.23 (3)
	(10nM)	9.08 ± 0.12 (3)	10.92 ± 0.20 (3)
	(100nM)	8.62 ± 0.17 (6)	10.98 ± 0.10 (6)
	Testosterone		
	(10nM)	9.16 ± 0.20 (3)	10.90 ± 0.09 (3)
	(100nM)	8.91 ± 0.16 (6)	10.61 ± 0.03 (6)
	Trenbolone		
	(1nM)	8.97 ± 0.05 (3)	10.53 ± 0.05 (3)
	(10nM)	8.84 ± 0.15 (3)	10.46 ± 0.11 (3)
	(100nM)	9.31 ± 0.18 (6)	10.58 ± 0.07 (6)
18h	None	21.05 ± 0.22 (4)	22.65 ± 0.22 (4)
	Trenbolone		
	(100nM)	20.73 ± 0.28 (4)	21.94 ± 0.15 (3)

Discussion

Although quite long measurement periods are frequently used in protein-degradation studies, it is evident from the results reported here that very different conclusions can be drawn from long

incubations as compared with relatively brief 4h measurement periods. The most pronounced difference is an observed inhibition of protein breakdown by foetal-calf serum if a brief measurement period is chosen, compared with a stimulation of the pathway if the evaluation period is 12h or longer. There is a gradual decrease in the rate of protein breakdown in the absence of serum or growth factors, whereas in their presence degradation proceeds at an almost uniform first-order rate. It seems likely that in Minimal Essential Medium without growth factors, degradation decreases because, over long periods, an increasing number of cells separate from the monolayer and lose viability. Accordingly the long measurement times are only appropriate under growth conditions or perhaps when protein synthesis and accumulation are being evaluated in addition to protein degradation.

Most of the effectors tested here produce rapid changes in protein breakdown which are maximal 2h after addition. However, dexamethasone requires several hours for its effect to develop, a result reported previously for hepatocyte monolayers (Hopgood *et al.*, 1981). Other agents known or suspected of gradually influencing muscle protein breakdown *in vivo* were not effective in L6 myoblasts. This group of compounds includes testosterone, diethylstilboestrol and trenbolone. It has been suggested that anabolic steroids and related compounds such as trenbolone act by interfering with the catabolic response of glucocorticoids on muscle (Mayer & Rosen, 1975; Heitzman, 1979). Although a catabolic response of dexamethasone was clearly evident in L6 myoblasts, we were unable to show any effect of the anabolic agents, either individually or in the presence of dexamethasone.

The lack of any response to testosterone, diethylstilboestrol or trenbolone does not reflect an insensitivity of confluent monolayers of L6 myoblasts towards all growth-promoting agents. Thus foetal bovine serum or colostrum substantially inhibited protein breakdown in L6 myoblasts, even when included at very low proportions in the medium. The active ingredients in these complex mixtures are not known, although serum is a rich source of insulin-like growth factors, agents that have been shown to inhibit protein breakdown with sensitivities appropriate to their content in serum (Ballard *et al.*, 1980*a*,*b*). Insulin certainly inhibits protein breakdown in the myoblast line studied here, but it is only effective at high concentrations. It seems likely that the insulin effect may be mediated via the hormone binding to receptors specific for insulin-like growth factors, as has been argued for other cell lines (Zapf *et al.*, 1981; Poggi *et al.*, 1979). Growth in L6 myoblasts is stimulated by multiplication stimulating activity (Richman *et al.*, 1980), a rat factor probably synonymous with insulin-like growth factor 2. Certainly the insulin contents of the foetal bovine serum and bovine colostrum used here, 50pM and 2nM respectively (Ballard *et al.*, 1982). are several orders of magnitude lower than is necessary to account for the inhibitory effects of the mixtures on protein breakdown in myoblasts. This result contrasts with experiments on H35 and MH_1C_1 hepatomas, two cell lines that are exquisitely sensitive to insulin, where virtually all the effect of bovine colostrum on protein breakdown can be accounted for by the insulin present (Ballard *et al.*, 1982). It is perhaps relevant that protein breakdown in these hepatomas is very insensitive to the insulin-like growth factors (Ballard *et al.*, 1980*a*).

The inhibition of protein breakdown in myoblasts by growth factors could be related to anabolic effects of testosterone and related agents on muscle *in vivo*. If such agents result in increased concentrations of somatotropin (growth hormone) or the insulin-like growth factors (somatomedins), as has been suggested (Wangsness *et al.*, 1981), an inhibition of muscle protein breakdown and a stimulation of protein synthesis might result. The present results are consistent with such an interpretation because it does not depend on a direct effect of the steroid or steroid-like anabolic agents on muscle protein turnover.

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